PATENT COOPERATION TREATY

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PCT

NOTIFICATION CONCERNING DOCUMENT TRANSMITTED

To:

United States Patent and Trademark Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as elected Office

Date of mailing (day/month/year)	
15 March 1996 (15.03.96)	
International application No.	**International filing date** (day/month/year)
PCT/NL95/00108	21 March 1995 (21.03.95)
Applicant	
RIJKSUNIVERSITEIT UTRECHT et al	

The International Bureau transmits herewith the following documents and number thereof:

_____ copy of the international preliminary examination report (Article 36(3)(a))

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer C. Carrié
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 730.91.11

Form PCT/IB/310 (July 1992)

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
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Date of mailing (day/month/year) 30 November 1995 (30.11.95)	
International application No. PCT/NL95/00108	**Applicant's or agent's file reference** BO 39207
International filing date (day/month/year) 21 March 1995 (21.03.95)	**Priority date** (day/month/year) 21 March 1994 (21.03.94)
Applicant ANDERTON, Stephen, Mark et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 October 1995 (04.10.95)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Peggy Steunenberg
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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:
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Scheveningseweg 82
2502 LS Den Haag
PAYS-BAS

WRITTEN OPINION

(PCT Rule 66)

Applicant's or agent's file reference: BO 39207	Date of mailing (day/month/year): 12.12.95
	REPLY DUE within 2 months/days from the above date of mailing

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NL 95/00108	21/03/1995	21/03/1994

International Patent Classification (IPC) or both national classification and IPC
C07K14/35

Applicant
RIJKSUNIVERSEIT UTRECHT et al.

1. This written opinion is the first (first, second, etc.) drawn up by this International Preliminary Examining Authority.

2. This report contains indications and corresponding pages relating to the following items:

I [X] Basis of the opinion

II [ ] Priority

III [X] Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

IV [ ] Lack of unity of invention

V [X] Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

VI [ ] Certain documents cited

VII [X] Certain defects in the international application

VIII [X] Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. The applicant may, before that time limit expires, request this Authority to grant an extension, see Rule 66.2(d).

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rule 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4*bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 21/07/1996

Name and mailing address of the IPEA	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Examiner: S-E Korsner S.-E. Korsner Formalities officer (incl. extension of time limits): Françoise Pannetton Telephone No. 8403

Form PCT/IPEA/408 (cover sheet) (January 1994) (22/11/1995)

Intern. application No.

WRITTEN OPINION

PCT/NL95/00108

I. Basis of the opinion

1. This opinion has been drawn up on the basis of (Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".):

[x] the international application as originally filed.

[ ] the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,

[ ] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,

2. The amendments have resulted in the cancellation of:

[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This opinion has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

WRITTEN OPINION

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

[ ] the entire international application,

[x] claims Nos. 1-3, 7-17 (when dependent on Claims 1-3)

because:

[ ] the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international preliminary examination (specify):

[ ] the description, claims or drawings (indicate particular elements below) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (specify):

[ ] the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

[x] no international search report has been established for said claims Nos. 1-3, 7-17(partially)_____.

WRITTEN OPINION

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-17 NO_____
	Claims _____
Inventive Step (IS)	Claims 1-17 NO_____
	Claims _____
Industrial Applicability (IA)	Claims _____
	Claims _____

2. CITATIONS AND EXPLANATIONS

The following documents will be referred to in this opinion:

D1 = WO-A-88065916
D2 = EMBO Journal, 1987, pp. 1245-1249
D3 = Journal of Immunology, 1988, pp. 2749-2754
D4 = EP-A-322 990
D5 = EP-A-262 710
D6 = WO-A-9010449

Novelty (Article 33(2) PCT)

The peptide sequences as claimed, including the more restricted ones of Claim 6, are anticipated by the documents D1-D3.
See especially the following pages:

D1, p.8.....sequence 231-245,
D2, p.1247...sequence 112-132, overlap in DDVAG = sequence

81-85 as claimed - see comment on page 1248, column 2, about identity.
See also the selection method, page 1248, column 2, bottom,

D3, Table 1..sequences 231-245, 241-255 and 91-105 - the latter overlapping with sequence 84-95 of Claim 6.

Since the related matter of the dependent claims is evident to the skilled man, no presence of novelty can be acknowledged at this stage.

Inventive step (Article 33(3) PCT)

Similar approaches using (other/overlapping) fragments of M. tuberculosis are already known in the prior art - see also the additional documents D4-D6, more specifically:

D4, p.6.....Use of other fragments for protection against induction of adjuvant arthritis,
D5, p.3.....Use of further fragments, including the neighbouring 171-240, for the preparation of compositions for alleviation, treatment and diagnosis of autoimmune diseases.
D6, p.7.....Use of fragments for prevention or treatment of diabetes mellitus.

Having regard to the teaching and extensive background references of the cited documents, it is evident that most of the characteristics of the hsp65 (and related proeins) are well known.
Moreover, the discussion of D3, pages 2752-53, 1988, is also instructive about the possibily offered by these peptides.

The Applicant is th refore ask d to restrict his claims, if possible with r gard to A34 (2)(a), and provide further information about the inventiv step [= further contribution to the art].

Since the concept can no longer be considered novel (i.e. other sequences have already been suggested), a non-unity objection may arise from the selection of different peptide sequences.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

1. Prior art (Rule 5.1(a)(ii) PCT)

To meet the requirements of Rule 5.1, the documents D1-D3 and D6 should be cited in the Description (depending on the future restrictions).

WRITTEN OPINION

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1.
On page 5 (bottom) and page 6 (top), the references to Fig. 14 are unclear, because said figure is not present.

2.
Since M. tuberculosis and M. bovis are not identical, the bracket of Claim 2 is not clear.
Should the complete sequence be identical, this should be pointed out.

3.
In Claims 5-7, it is not clear whether the wording "..comprises at least 5 amino acids which are in the same relative position.." indicates identical amino acids or not.

4.
Claim 9 has no clear interpretation, because the exchange of one or, in particular, more amino acid(s) may result in a completely different peptide, especially in the case of shorter peptides.

5.
Claim 10 borders on a scientific theory under Rule 39(i), and there is no evidence that this will invariably lead the skilled man to a successful result.
In view of the anticipating prior art, the question of non-unity also arises.

6.
Claim 13 appears to include known microorganisms; in the case of special strains, see Rule 13bis PCT (deposits).

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/35, C12N 9/02, 15/31, 15/53, 15/63, 5/08, C07K 16/18, A61K 38/08, G01N 33/68

A1

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94202927.3 10 October 1994 (10.10.94) EP
(34) Countries for which the regional or international application was filed: AT et al.

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PEPTIDE FRAGMENTS OF MICROBIAL STRESS PROTEINS AND PHARMACEUTICAL COMPOSITION MADE THEREOF FOR THE TREATMENT AND PREVENTION OF INFLAMMATORY DISEASES

(57) Abstract

Peptides are provided which are useful for protection against or treatment of an inflammatory disease, including autoimmune diseases, such as diabetes, arthritic diseases, atherosclerosis, multiple sclerosis, myasthenia gravis, or inflammatory responses due to tumour or transplant rejection. The peptides contain a part of the aminoacid sequence of a microbial protein having a conserved mammalian stress protein homologue, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 25 %, and the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 30 %, said part comprising at least 5 aminoacids which are in the same relative position as the same aminoacids in a T cell epitope of said stress protein, which epitope contains at least 4 consecutive aminoacids which are identical with the corresponding mammalian stress protein aminoacids. Nucleotide sequences, expression systems, antibodies and pharmaceutical and diagnostic compositions derived from these peptides are provided as well.

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Your ref. International Preliminary Examination Authority
Our ref. BO 39207 RJ/MM

The Hague, Febaruary 12, 1996

Re: International Patent Application No. PCT/NL95/00108
in the name of Universiteit Utrecht et al.

Dear Sir,

This is in response to the Written Opinion dated 12 December 1995.

Novelty:

It is believed that the peptide sequences as claimed are not anticipated by document D1-D3, nor by any other prior art document.

In considering novelty of the subclaims, such as claim 5 or 6, it should be borne in mind that they do not just specify a part of the *M. tuberculosis* hsp60 protein as defined by their amino acid numbers, but also by the criteria which are laid down in the claims on which these subclaims depend. In particular, the criteria of claim 1 state that the partial amino acid sequence should at least contain 5 amino acids of a T cell epitope of the microbial stress protein and that said epitope contains at least 4 consecutive amino acids which are identical between the mammalian and the microbial proteins.

The sequence of D1, page 8, 231-245, does not comply with the criteria of the claims, even though it shares 5 amino acids with the sequence mentioned in claim 5, because it does not contain at least 4 consecutive amino acids which are identical between mammalian and microbial protein. This can be seen in the present figure 13, which shows that in the sequence 231-245 (lowest line of p. 1 of fig. 13) there are only 3 consecutive identical amino acids. In addition, the sequence 231-245 of D1 does not contain a T cell epitope, or at least is not said to have such an epitope.

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Similarly, the sequence 112-132 of D2, page 1247 does not anticipate the sequence of claim 5, or any other claim, because there is no series of 4 consecutive identical amino acids. Also, the method of selecting the peptides according to D2 is completely different from the present method (compare D2, p. 1248, last lines with patent claim 10). The sequences of Table 1 of D3 do not anticipate the present sequences either, even though they partly coincide with the sequences as numbered in claim 5, because sequences 91-105 and 241-255 do not comprise a T cell epitope as follows from the table itself, and sequence 231-245 does not contain 4 consecutive identical amino acids as explained above.

Although it is not believed to be relevant to the question of novelty, the Examiner's opinion that the "related matter of the dependent claims is evident to the skilled man" is not understood. For example, no prior art document appears to even remotely suggest the feature of claim 8.

Inventive step:

The present invention concerns a systematic approach for the development of tools in the treatment and diagnosis of inflammatory diseases, including autoimmune diseases. The inventors believe that they are the first to have provided such a systematic approach, the approach being the use of proteins similar but not identical to the mammalian self-proteins related to the particular disease. This approach has resulted in a method of selecting suitable peptides (present claim 10), the peptides themselves described by well-defined criteria (claims 1-9) as well as further tools derived therefrom (claims 11-17).

Therefore, the opinion that "similar approaches using fragments of *M. tuberculosis* are already known in the prior art", is believed not to be correct.

It may be true that several fragments of *M. tuberculosis* have already been described and been claimed to have a particular use, but these previously described fragments do not meet the definitions of the peptides according to the inventions, and, moreover, they do not have the desired function and thus are of no use in the aim of the present invention. This has already been explained above where the fragments described in D1-D3 are concerned, but the same applies to the fragments mentioned elsewhere:

D4 proposes polypeptides corresponding to the sequence 172-192 of *M. bovis*, from which the sequence 172-179 and/or 179-192 should be absent. The only part of the hsp60 sequence 172-192 that might meet the present criteria is the sequence 171-175 (see fig. 13(1), numbering of M.tub), but this sequence should precisely be absent according to D4.

Our ref. BO 39207 RJ/MM Febaruary 12, 1996

D5 proposes a larger sequence, which does not comply with the present criteria either. Moreover, it is described in the present application (page 18, 19 and figures 7 and 10) that an immune response against epitopes in this region does not induce protection and, furthermore, the epitopes in this region do not meet the selection criteria of the present invention.

The fragment disclosed in D6 (see claim 32 thereof) corresponds to sequence 410-430 of the *M. tuberculosis* hsp60 which, again, does not fall under the present definitions, for instance because it does not have the required 4 consecutive identical amino acids.

It may be true that many characteristics of hsp65 are known, but there is no scientific basic whatsoever for the assumption that "most of the characteristics of hsp65 are well known". In contrast, it is believed that we are only at the beginning of understanding some of the characteristics and functions of hsp65 and other stress proteins.

D3 may be instructive as to the possibilities offered by these peptides, but the peptides disclosed therein are the wrong peptides according to the present invention, and moreover, D3 is perhaps instructive for diagnosis and protection in mycobacterial infectious diseases, but certainly not for autoimmunity.

The applicant is of course willing to provide further information about the inventive step, possibly in an informal interview, but after the explanation here above it is not yet clear to them what the remaining objections as to inventivity could be. In anticipation a copy of a publication by the inventors, which is presently in press, explaining in more detail the merits of the invention, is enclosed herewith. The most relevant parts are underlined.

Prior art:

Substitute pages for page 2 are enclosed herewith, containing a reference to D1-D3 and D6.

Remaining issues:

1. Figure 14 should be present, because it was filed together with the other documents of the application. Fig. 14 followed the hard-copy version of the sequence listing, and did not directly follow fig. 13. As a result, the sheets of figure 14 were inadvertently not counted in the sheets of the drawings in box VIII of the request form, but they were contained in the sequence listing. The reason is probably that figure 14 was filed in the third priority application as a sequence listing, and was converted to a revised sequence

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listing and a figure after a formality requirement by the European Patent Office. An additional copy of fig. 14 is enclosed herewith.

2. In the amended claims filed herewith, claim 4 (not claim 2) has been amended so as to clarify the identity between the two proteins.

3. The identity of the at least 5 amino acids has been clarified by amendments of claims 1, 5, 6 and 10.

4. It cannot be seen how the feature of claim 9 could result in a completely different or a shorter peptide. The wording of the claim, and if necessary, page 8, lines 16-32, make it clear that the peptide cannot be completely different or shorter.

5. Although claim 10 may be partly based on a scientific theory, the claim itself contains clear-cut, concrete measures to be taken by the technicians in order to arrive at the desired result. If necessary, the exemplification on page 5 line 29 - page 7, line 5 together with figure 14 and/or Seq. No.'s 2 and 3 will give further unambiguous guidance.

6. Since claim 10 ultimately depends on claim 1, and claim 1 has been amended so as to specify that the peptide contains no more than 30 peptides, claim 13 appears not to comprise known microorganisms.

Apart from the amendments already referred to above, the amended claims filed herewith also contain amended claim 1, wherein the minimum homology of the sequence of 75 amino acids is put at 40%, and the total length of the peptide sequence is restricted to 5-30 amino acids (original claim 7), and the presence of 4 consecutive amino acids is clarified; as well as an amended claim 17. Amended parts are underlined in one copy of the claims.

It is requested that in case major objections as to novelty or inventive step should remain, an opportunity for further explaining the merits of the invention, be it in writing or orally, be given before the International Preliminary Examination Report is drawn up.

Yours sincerely,
Nederlandsch Octrooibureau

R. Jorritsma

Encl.: copy publication, substitute pages for p. 2, claims, copy fig.14

Immunological Reviews 1995. No. 148

Immunological Reviews
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(Altered) Self Peptides and the Regulation of Self Reactivity in the Peripheral T cell Pool

WILLEM VAN EDEN, STEPHEN M. ANDERTON*, RUURD VAN DER ZEE, BERENT J. PRAKKEN, CHRIS P. M. BROEREN & MARCA H. M. WAUBEN

INTRODUCTION

Thymic negative selection is a mechanism by which potential autoreactive T cells are eliminated. However, cellular techniques of T cell cloning have revealed the presence of self-reactive T cells in the repertoire of the healthy individual. Now it is widely assumed that peripheral tolerance is an important mechanism to regulate the potential self reactivity of the latter T cells. Therefore, it is possible that the positively selected repertoire harbours T cells that have the capacity to support T cell regulatory mechanisms of peripheral tolerance.

Models of induced autoimmune diseases have shown that the autoimmune potential of the healthy immune repertoire can be unleashed under experimental conditions. Many self antigens, when integrated into a suitable adjuvant such as complete Freund's adjuvant (CFA) or non-mycobacteria containing mineral oils (IFA), have been shown to break tolerance upon experimental immunisation. It seems as if in that case, aggression (Th1?) promoting conditions serve the purpose of tipping the system over the edge into developing overt pathological self reactivity. Autoimmune arthritis, which may possibly be regarded as an example of TH1 dominated immunopathology, can be induced in animals by using a variety of oily adjuvants,

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Correspondence to: W. van Eden MD PhD, Utrecht University, Faculty of Veterinary Medicine, Institute of Infectious Diseases & Immunology, Department of Immunology, Yalelaan 1, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands.

even without the addition of self antigen such as collagens and proteoglycans. This suggests that under such conditions virtually any self antigen present at the site of immunisation is capable of triggering "aggressive" responses in T cells with the suitable specificity of seeing the self antigen in joints. In addition, it is possible that exposure of the system to self antigens may also help to reinforce the control of self reactive cells. This can occur during the spontaneous remission of the induced disease or by exposing the immune system to the self (auto-)antigen in the absence of aggression-promoting conditions. The latter may be the case for heat-shock proteins in arthritis.

Immunisation of rats with whole mycobacteria, which may be an essentially Th1 promoting condition, leads to arthritis. The same form of arthritis has been shown to be transferable using a T cell clone recognising mycobacterial hsp60. However, upon immunisation with the mycobacterial protein hsp60 in the absence of mycobacteria, no arthritis is seen and all that remains is increased resistance to subsequent induction of arthritis, no matter which substance is used for arthritis induction. Evidence has been collected now that such protection may have resulted from the induction of self hsp60 cross-reactive T cells (see below; Anderton et al. 1995a). Supportive evidence that similar mechanisms may be operative in natural autoimmune diseases comes from children with chronic rheumatoid arthritis. It appeared that T cell responsiveness to human hsp60 in these patients is associated with a remitting course of their disease. T cell responses were not seen in patients with non-remitting disease. Interpretation of these experimental observations, has led us to propose that T cell responses directed at self hsp epitopes are part of regulatory mechanisms contributing to dominant forms of disease-suppressive peripheral tolerance.

For the immune system, heat-shock proteins are likely to be a special case for several reasons.

First of all, hsp's are exceptionally well conserved proteins, which is reflected by the presence of a considerable degree of sequence identity between bacterial hsp's and their mammalian counterparts.

Secondly, despite their similarity with self antigens, hsp's are exceptionally immunogenic bacterial proteins. This may be due to repeated or continuous exposure of the immune system to such proteins during vaccination and infection or by contact with the residential intestinal flora. Alternatively, since heat-shock proteins appear to have a high level of expression in thymic cortical cells, the site in the thymus where positive selection occurs (Birk et al., in press), mechanisms of thymic positive selection could have created a relative overrepresentation of hsp reactive cells, with the capacity to cross-recognise conserved bacterial hsp epitopes. And, indeed, specificity analysis of hsp reactive cells has revealed that, under *in vitro* conditions, these cells are sometimes also responsive to self (mammalian) hsp's. The question that remains is how, despite their abundant presence, such self hsp responsive cells are kept functionally tolerant. A possibility is that such tolerance is maintained through the continuous interplay of self hsp responsive T cells with bacterial hsp's at the mucosal surfaces of the gut. Here the cells are confronted with

bacterial hsp's and the system is conceivably tuned to avoid inflammatory responses. As we know, presentation of antigens at the gut mucosa is a preferred route of tolerance induction. Thus, tolerance for self hsp's may well depend, at least in part, on the principles of mucosal induced tolerance. The finding that the presence of intestinal flora contributes to resistance to experimental autoimmunity in various models, can be seen as being supportive of this possibility. In this way also exposure of the immune system to bacterial hsp at the mucosal surface may contribute to expanding a hsp reactive repertoire, although according to principles of oral tolerance such a repertoire may behave in a functionally different (tolerant?) way. Furthermore, with self hsp's being close to their bacterial analogs, but not identical with these, effects such as those seen with altered peptide ligands at the clonal T cell level may be operational in maintaining a relative state of functional tolerance in self hsp specific cells, upon recognising self hsp, and perceiving it as a peptide analog of the bacterial hsp epitope. The work by Wauben et al. (1992a) has shown that single amino acid substituted peptides based on protein sequences of autoimmune disease associated molecules, are capable of dramatically reducing the incidence and severity of induced autoimmune diseases. Based on observations in preimmunisation experiments and by comparison of relative MHC binding capacity with disease suppressive potential, it has become clear that such peptide analogs have activities that exceed mechanisms of MHC blocking. Apparently, subtle alterations in protein sequences of disease-associated antigens may yield peptides with the potential of stimulating mechanisms of disease-controlling peripheral tolerance. It is possible that during thymic selection, where full agonistic interactions may lead to apoptotic cell death, positive selection of T cells is operative through recognition of the self antigens in the form of partial agonists (Jameson et al. 1995). In the case of hsp reactive cells, such cells, selected by recognising self hsp peptides as partial agonists, may then have undergone expansion in the periphery on the basis of recognition of bacterial hsp's acting as full agonists for these cells. Upon recognition of target self hsp, such recognised self peptides would act again as partial agonists, setting the responding cell in a non-responsive, tolerance promoting, mode. Even so, when self hsp's are expressed at the site of inflammation (stress response) it is possible that such mechanisms do contribute to dominant (bystander) regulation, leading to suppression of the inflammatory response.

A *third* characteristic aspect of hsp's is their regulated, stress-dependent level of expression in tissues. Their raised synthesis during any stressful event, including inflammation, makes hsp's dependable antigens that will be expressed where the action is. In that sense, taking into account that self hsp's may serve as targets for self-directed regulation, hsp's could be ideal molecules signalling feed-back down-regulatory events in the case of (autoimmune?) inflammation. Their low level of expression under normal non-stressed situations may be an additional critical factor in the maintenance of tolerance with regard to these proteins. Low expression levels under normal conditions would take away the necessity of tolerance for such antigens to be complete under all circumstances.

Regulatory events in peripheral T cells, contributing to peripheral tolerance, can be based on peripheral bystander suppression, where tolerance is maintained through the elaboration of suppressive cytokines that control activated cells in their immediate vicinity. In addition direct T–T cell interactions among activated cell populations may play an important role. Broeren et al. (1994) have isolated TcR V gene specific T cells, with the capacity of directly recognising the relevant activated T cell clone. Moreover, such T cells were found to exert disease suppressive activities in experimental arthritis. In this case, the raised expression of MHC class II molecules on activated T cells, together with internalisation of T cell receptors following triggering of the cells, was suggested to offer possibilities for MHC restricted presentation of clonotypic markers of T cells to other T cells with regulatory capacity as a feed-back mechanism of peripheral tolerance that becomes active after T cell activation.

ADJUVANT ARTHRITIS AS A MODEL FOR T CELL REGULATION OF AUTOIMMUNITY

One of the best studied models of arthritis is AA. Disease is induced by the immunisation with mycobacterial antigens and is transferable by T cells alone. Holoshitz et al. (1983) have shown that passive transfer of a single T cell clone, being reactive to mycobacteria can also lead to induction of AA. Subsequent analyses have shown that this arthritogenic T cell clone, called A2b, recognised the 180–188 sequence in mycobacterial hsp60 (Van Eden et al. 1988) and also responded, although to a lower extent, to cartilage proteoglycan (Van Eden et al. 1985, 1989). This was the first demonstration of the fact that autoimmune T cells, with a single antigenic, in this case "self" cross-reactive, specificity, can induce the full pathology of clinically overt arthritis. Further experiments have shown that the same, but attenuated, T cells and their specific antigens (or epitopes) in the form of synthetic peptides, can be used to induce a specific form of protection against AA.

CONSERVED BACTERIAL HEAT-SHOCK PROTEIN PEPTIDES INDUCE DISEASE-SUPPRESSIVE REGULATORY T CELLS

Hsp60 was defined as an arthritis-associated antigen by the fact that arthritogenic A2b, raised against whole *M. tuberculosis* in the AA model, responded to recombinant mycobacterial hsp60 as cloned in *E. coli* (Van Eden et al. 1988). Having identified mycobacterial hsp60 as a critical antigen in the induction of AA in Lewis rats, experiments were initiated to see whether arthritis was inducible by immunisation with recombinant hsp60. However, no arthritis was seen to result from hsp60 immunisation and immunised animals were found to have become resistant to subsequent induction of AA using whole mycobacteria. Mycobacterial hsp60 was also found to protect against streptococcal cell-wall induced disease, pristane (oil) induced disease and to a variable degree also against avridine and collagen type II in-

duced arthritis (reviewed in Van Eden et al. 1989, 1995, Van Eden 1991). This seemingly general activity of mycobacterial hsp60 in preventing experimentally induced arthritis also in models not induced with mycobacteria was compatible with the possibility that "self" hsp60 was an autoantigen critically involved in every form of autoimmune arthritis.

As reviewed elsewhere (Welch 1993), heat-shock proteins (hsp's) are intracellular proteins having critical functions in the protein house-keeping machinery of every living cell. Probably due to such essential cellular functions, their evolutionary variation has remained remarkably limited. As a consequence, extensive amino acid sequence identities exist between microbial and mammalian hsp's. Despite their similarities with self (host) hsp's, microbial hsp's have been found to be strong immunogens. Therefore, the possibility existed that immunisation with mycobacterial hsp60 led to induction of responses directed to self hsp60 as an autoantigen.

An important feature of hsp's is their raised synthesis when cells are subjected to stress (they are also called stress proteins). Inflamed synovium, apparently a situation characterized by local cellular stress, was shown to feature a more intense staining in the immunohistology using hsp60 specific antibodies than healthy synovium (Boog et al. 1992). In the experimental arthritis models, not only was such raised expression seen, but also T cell reactivity against hsp60 was observed in diseased animals, irrespective of how arthritis was induced. Thus, altogether the observations made in various models suggested that the presence of arthritis led to immune responses directed to endogenous self hsp60 as expressed in the inflamed synovium. Furthermore, evidence was obtained that (myco)bacterial hsp60 immunisation led to enhanced hsp60 responsiveness which could possibly contribute to protection against arthritis.

Recent observations in the AA model are supportive of this hypothesis (Anderton et al. 1994, 1995a,b). Lewis rats were immunised with either whole *M. tuberculosis* or recombinant mycobacterial hsp60 in a suitable adjuvant. Primed lymph node cells, taken from immunised rats, were analysed for their proliferative responses in the presence of overlapping peptides spanning the whole mycobacterial hsp60 molecule. It turned out that immunisation with whole *M. tuberculosis* (Mt) led to responses to various peptides indicating several epitopes located on the hsp60 molecule. However, responses to a peptide that included amino acids 180–188, formerly demonstrated to be the epitope recognized by arthritogenic T cell A2b, were found to dominate the response pattern obtained.

In contrast, immunisation not with Mt but with the *protein* mycobacterial hsp60, led to responses to the same epitopes and to some additional epitopes, but interestingly no dominance of 180–188 was apparent. All epitopes involved were mapped in detail by generating epitope specific T cell lines (Fig. 1). Upon testing these T cell lines for responses to peptides based on homologous rat hsp60 sequences, only one T cell line (H_52) also responded, although to a lower level, to the homologous rat hsp60 peptide. As to be expected from the cross-recognition between the myco-

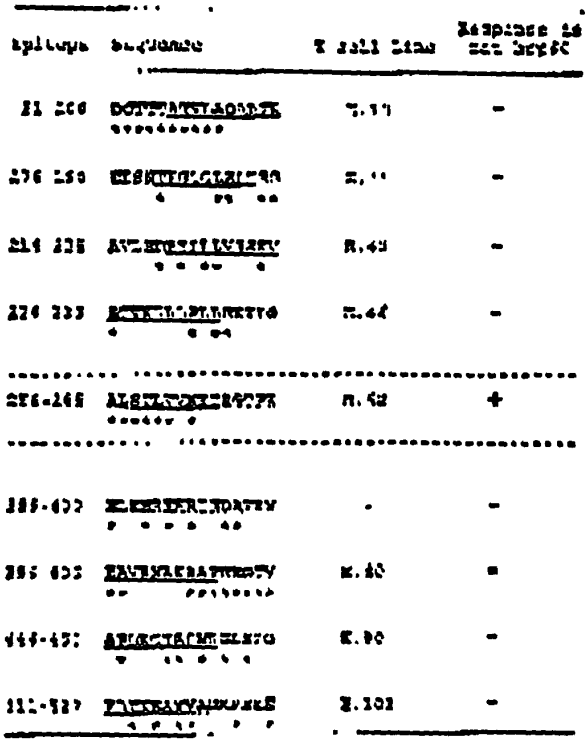

Fig. 1. Mycobacterial hsp65 (=hsp60) T cell epitopes in the Lewis rat. Sequence homologies with rat hsp60 are indicated. Identical residues are indicated by *. Underlined are the epitope core sequences. Only T cell H.52 was responding to rat hsp60 and protected against arthritis upon transfer.

bacterial and rat sequence, this particular epitope, which was the sequence 256–265, turned out to be strongly conserved (see Fig. 1) (Anderton et al. 1995a,b). Furthermore, the T cell was shown to recognise heat-shocked (after culture at 42°C for 1 hr) antigen-presenting cells (spleen cells) without addition of peptide. Transfer experiments showed that only this particular T cell conferred protection against disease, while all other hsp60 specific T cell lines tested did not. So, in this case, it was the autoreactive cell, which responded to self stress proteins, that suppressed disease (Anderton et al. 1995a).

The various peptides that included our newly defined T cell epitopes were tested for their capacity to protect Lewis rats against AA by immunisation prior to disease induction. Remarkably, only peptides containing the conserved, rat hsp60 cross-re-

active, 256–265 epitope were protective. These findings suggested that for induction of protection against disease, mycobacterial hsp60 was dependent on activity of T cell epitopes in the molecule, which activated T cells that cross-reacted with rat self hsp60.

More recent experiments have shown that non-mycobacterial induced arthritis, such as arthritis induced with the non-antigenic synthetic adjuvant Avridine (Chang et al. 1980) was also effectively prevented by preimmunisation with the conserved peptide. Teleologically arguing, it seems attractive to suppose that the cross-recognition of rat self hsp60 is the basis of the protection observed. Thus, the stimulation of responses to self hsp60 could lead into a regulatory (suppressive) response targeted to the expressed self hsp60 molecule. Such suppressive regulation may well contribute to the control of local inflammatory processes. Some of the findings made in children suffering from JCA seem to be compatible with these possibilities.

Responses to human hsp60 in children with chronic arthritis.

In humans, in the case of RA, disease is usually progressive, whereas in children disease will remit in many cases. Probably with the exception of very early cases of disease, proliferative responses to hsp60 are relatively low in the majority of patients with RA (Res et al. 1990), although non-proliferative responses, such as production of certain cytokines after stimulation (Wilbrink et al. 1992, 1993) have been demonstrated in the presence of hsp60. In children with JCA, however, proliferative responses have been obtained in lymphocytes taken from both the peripheral blood and the synovial compartment. Consistent responses were seen by stimulating the cells with the self antigen human hsp60 (De Graeff-Meeder et al. 1991).

Since most of the patients, however, also responded to mycobacterial hsp60, it seems that also in this case, conserved epitopes, equally present in both the human and mycobacterial hsp60, are recognised by patient T cells. From comparison of patients of distinct clinical subgroups, it became evident that responders had oligoarticular (OA) forms of JCA, whereas non-responders had polyarticular or systemic JCA. In other words, those with a remitting form of disease responded, whereas those having a non-remitting form did not (De Graeff-Meeder et al. 1995). Furthermore, it was found that *in vitro* priming of non-responder cells, obtained from OA-JCA patients during phases of disease remission, led to positive secondary responses only in case of OA-JCA and not in the case of other clinical subgroups of JCA (Prakken et al., submitted). Longitudinal studies indicated that remission of OA-JCA was preceded by proliferative responses to human hsp60, that during remission proliferative responses were absent, and that a temporary clinical relapse was followed by reappearance of proliferative responses.

Altogether, the data obtained in JCA patients have shown that responses to human hsp60 as a self antigen do occur and that they are associated with relatively benign forms of arthritis. In this sense, it may be surmised that the presence of such

responses protects these patients from developing a non-remitting chronic form of disease. The presence of responses during the active phase of disease, preceding remission, suggests, in line with the observations made in the AA model, that responses to self hsp60 may positively contribute to mechanisms leading to disease remission. If so, possibilities for immunological intervention in JCA, and possibly also RA, may be found in strategies aimed at manipulating peripheral tolerance through vaccination with hsp60, or peptides containing defined conserved hsp epitopes.

The superior disease suppressive potential of bacterial hsp over mammalian hsp.

The possibility that the presence of bacterial flora contributes to the establishment or maintenance of peripheral tolerance has been suggested by experimental findings in germ-free animals. In the case of arthritis, experiments performed in inbred Fisher rats have been most illustrative (Kohashi et al. 1986). Fisher rats are relatively resistant to the induction of arthritis following immunisations with mycobacteria or streptococcal cell walls. However, germ-free bred Fisher rats are susceptible to a degree similar to Lewis rats. Reconstitution of the gut with *E. coli* bacteria or with the bacterial flora as present in conventionally bred animals was shown to lead to arthritis resistance, despite the fact that the animals had a history of germ-free development. Altogether, it seems that mechanisms of peripheral tolerance as a hedge against autoimmunity are dependent, at least in part, on interactions with the environmental microflora.

To achieve a lasting restoration of tolerance in the case of disease, it seems most adequate to target immunotherapy to the enforcement of natural mechanisms that contribute to maintenance of self tolerance. In other words, exposure of the immune system to cross-reactive bacterial antigens, such as hsp's, might well stimulate the immune system to resume control over unwanted self-reactive clones. In line with the known contribution of bacterial gut flora to tolerance, it seems best to effectuate such exposure through oral administration of bacterial antigens. Although little support for the effectivity of such an approach can be obtained from work in experimental disease models, so far from experience in human medicine such support can be obtained. Laboratoires-OM (Geneva) has been producing *E. coli* bacterial lysates, which are used amongst others for the treatment of RA. They are administered via the oral route and have shown in multiple trials in RA patients an effectivity comparable with that of gold (Rosenthal et al. 1991). Recent analyses have revealed that the *E. coli* hsp60 molecule (GroEL) is one of the more prominent immunogens present in this material (Vischer & Van Eden 1994). This would suggest that *E. coli* hsp60, when administered orally, may trigger a T cell regulatory event that contributes to the control of RA, in a way similar to the effect of mycobacterial hsp60 in models of experimental arthritis.

As discussed before, the conserved bacterial hsp60 sequence 256–265 was found to induce protection against arthritis induction, most likely through the induction of

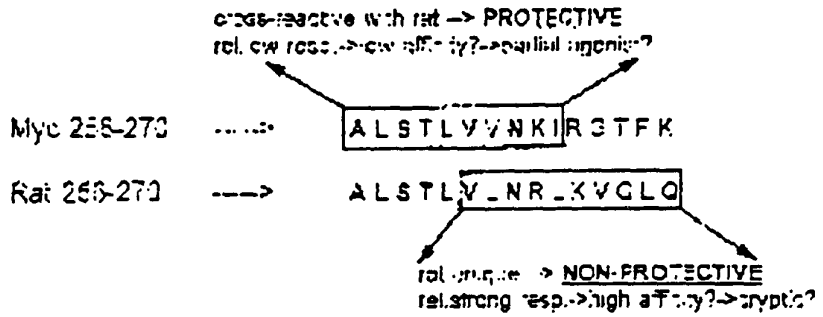

Fig. 2. Rat peptide 256–270 fails to induce protective T cells. Upon immunisation with rat 256–270 proliferative T cell responses were detected with specificity for a cryptic rat-unique non-conserved part of this peptide.

self hsp60 reactive T cells. However, upon immunisation with a longer peptide comprising the equivalent rat hsp60 256–270 sequence, the "rat analog" peptide, the animals were not found to be protected. Detailed analysis of the T cells responding to the rat (self hsp) peptide, revealed that such cells were recognising the carboxy-terminal *non-conserved* part of the peptide (see Fig. 2). Moreover, the cells were not proliferating in the presence of the complete mammalian hsp60 molecule. In other words, being confronted with this self peptide, the animal appeared to have selected for recognition a cryptic epitope. Thus, as far as we can infer from the data obtained with the hsp60 256–270 sequence, there is evidence that protection is obtained more easily with bacterial (self-like) sequences than with the self sequence itself. The responses of the bacterial hsp60 256–265 specific cells in the presence of the self hsp60 256–265 analog were relatively low compared to the response induced by the bacterial sequence. Thus, it is possible that the cross-reactive self response is due to the presence of low-affinity self-reactive T cells. Such cells may well have escaped the process of negative selection, and may have been positively selected, and such cells may be of a regulatory nature in the presence of this same self antigen. In that case, it is possible that such self epitope is seen by the cells in the periphery as non-stimulatory peptide analogs.

In line with this are recent experiments done with human (RA) T cells, which have indicated the elaboration of functionally distinct cytokine patterns depending on whether the cells were stimulated with the mycobacterial or the human hsp60 as an antigen (van Roon et al., in preparation).

DOMINANT DISEASE SUPPRESSIVE POTENTIAL OF PEPTIDE ANALOGS

The remarkable potential of our self-like bacterial peptide and not the self peptide itself to induce disease protective regulation through T cell cross-recognition of

self, may be related to the phenomena as we and others have seen using peptide analogs of "disease-associated" T cell epitopes. Various immune response modifying activities of such peptide analogs have been described and named accordingly: Altered Peptide Ligand (Evavold et al. 1993), Competitor-Modulator (Wauben et al. 1992b), Antagonist (DeMagistris et al. 1992) and Partial Agonist (Racioppi et al. 1993). Some years ago already, we ourselves introduced the term "Competitor-Modulator" peptides. This for their capacity on the one hand to block MHC presentation of these "dangerous" epitopes by means of superior MHC binding qualities and on the other hand to modulate responses with specificity for these "dangerous" epitopes by means of their immunogenicity and close antigenic relationship with the "dangerous" epitopes (Van Eden et al. 1993). In the model of adjuvant arthritis, a peptide based on the 180–188 sequence of mycobacterial hsp60 with a change of the Leucine at position 183 into an Alanine residue (called A183), was found to have a unique capacity to prevent development of AA when administered at the time of Mt immunisation for arthritis induction (Wauben et al. 1992a). The modulator aspects of the same peptide became apparent when preimmunisation with the same peptide one week before arthritis induction was found to inhibit the development of arthritis.

At the same time we have been testing a peptide analogue of a rat (also Lewis) EAE associated MBP T cell epitope. Despite superior MHC (RT1-B-^{l}) binding affinity and EAE inhibiting activity upon coimmunisation with MBP in rats, the MBP analog did not inhibit AA when tested at concentrations where A183 was active in suppressing AA. Especially the latter observations have pointed out that the disease suppressive potential of these peptide analogs must be based on "more than blocking alone". One possibility is that their activity is related to their capacity to trigger T cells with a specificity identical with or related to disease-inducing T cells. Such T cells could have regulatory potential. The Alanine 183 substituted 180–188 peptide was found indeed to trigger such T cells (T cell line ATL) with related fine specificity. The responding cells were found to cross-recognise the 180–188 peptide but not to proliferate in the presence of the whole hsp60 protein (Wauben et al. 1993). It is possible that this A183 peptide-induced T cell, despite its lack of capacity to proliferate in the presence of naturally processed hsp60, was capable of recognising the still elusive mimicked cartilage epitope. Upon recognition of this epitope in joints, this cell could possibly exert bystander suppressive activities (Friedman & Weiner 1994, Cohen 1995). On the other hand, such peptides could function as partial agonists or antagonists delivering a signal to the T cell receptor of the autoreactive T cell which induces a relative state of anergy or other altered response in the T cell. It is unclear at present whether or not such relatively anergic cells could contribute to *dominant* mechanisms of peripheral tolerance, for instance through consumption of cytokines such as IL2 or through competition for MHC-peptide complexes on the antigen-presenting cells (Lombardi et al. 1994) or again, by the elaboration of regulatory cytokines.

Apart from the immuno-modulatory effects of peptide analogs, also their compe-

tition for MHC binding in itself may contribute to the generation of functional changes in immune responses to antigens critical to disease. This has become apparent in recent experiments in which experimentally induced autoimmune myasthenia gravis (EAMG) in rats was found to be inhibitable by an OVA peptide with strong RT1-B1 binding capacity. From the distribution of isotypes of the developing anti-Acetylcholine receptor antibodies, the evidence was collected that in the presence of the MHC competitor peptide the response had shifted from Th1 in the direction of Th2 dominated responsiveness (Wauben et al., submitted).

Be that as it may, peptide analogs may have still not confessed all of their secrets. The dramatic impact of such peptides, as seen in distinct disease models, holds some promise for their use in treating autoimmunity. It is equally attractive to suppose that their great potential is already exploited naturally by the immune system not just to maintain peripheral tolerance, but more in general to establish and maintain a repertoire by seeing self as partial agonists while seeing non-self bacterial or viral epitopes as full agonists.

NASAL TOLERANCE TO HSP PEPTIDES SUPPRESSES ANTIGEN- AND NONANTIGEN-INDUCED EXPERIMENTAL ARTHRITIS

Recently, the unique relationship of the 180–188 sequence with the arthritic process was further substantiated by tolerising rats for this particular sequence by administering this peptide in the nose (nasal tolerance) or by giving it subcutaneously in PBS at high dosages (high dose tolerance). This procedure was seen to protect the animals from the subsequent induction of arthritis by either mycobacteria in oil or by the non-antigenic synthetic adjuvant avridine (CP20961) (Prakken et al., in press). Apparently, this single bacterial epitope, which resembles a (so far not identified) self epitope at the site of inflammation, is capable of inducing regulatory mechanisms of peripheral tolerance. The success of this regimen in suppressing disease, and especially in the case of non-microbially induced disease, seems to support the possibility that this microbial epitope, indeed, has a unique relationship with a disease-critical self antigen in the joint. Apparently, the exposure of such an antigen at the mucosal surface of the nose is already sufficient for setting reactive T cells in a regulatory mode, with the capacity to enforce peripheral tolerance leading to disease resistance.

T CELL RECEPTOR V GENE PEPTIDES INDUCE DISEASE SUPPRESSIVE T–T CELL INTERACTIONS

Another remarkably effective way of stimulating resistance against induced autoimmune diseases in various models is "T cell vaccination". T cell lines or clones.

with the potential of transferring disease, were irradiated or attenuated by other means and shown to transfer disease-specific resistance (Cohen 1986, 1989). Only recently, evidence was obtained that the T cell repertoire contains T cells with the potential of recognising processed T cell receptor V-gene products in the context of MHC molecules as expressed on activated T cells (Broeren et al. 1994, 1995b).

Arthritogenic T cell clone A2b has been shown to be effective in T cell vaccination regimens carried out in AA (Lider et al. 1987). Upon cloning and sequencing of the A2b TcR variable genes, A2b was found to use Vβ18 and Vα2. Based on this sequence, overlapping sets of peptides, spanning the full Vβ and Vα chain, were used to immunise rats. It appeared that a number of clustered peptides were highly immunogenic and elicited vigorous T cell responses (Fig. 3). Remarkably enough, the immunogenic TcR peptides were clustering within hypervariable CDRs of the TcR. immunisation of rats with these immunogenic peptides induced CD4+ TcR peptide specific T cells, which recognised in part their respective rec-DNA TcR proteins (Table I). Therefore, the presence of these responses could not be attributed to recognition of cryptic epitopes.

Furthermore, one of the lines generated, recognising TcR Vβ18 (17–31), recognised T cell clone A2b itself. This recognition was inhibited by antibodies against MHC-class II molecules, and anti-RT1-D^l (OX17) in particular (Table I). Therefore, it seems that activated T cells, when expressing MHC class II molecules on their surface, have some of these class II molecules filled with TCR V-gene product fragments and that such activated T cells may stimulate other T cells in a clonotype specific manner. The particular Vβ18 (17–31) fragment is in the CDR1 region and is therefore not strictly clonotypic. In other words the responding T cells have the possibility of responding to other clones, with distinct antigenic fine-specificities, which however do utilise Vβ18. In the case of AA this may be of relevance; when testing a large series of distinct T cell lines that were raised against peptides 180–188 or 176–190, almost all lines were expressing Vβ18, while lines directed against other peptides were Vβ18 negative. Therefore, Vβ18 CDR1 responding T cells could have multiple but rather specific interactions with the majority of the T cell populations in response to the arthritis associated epitope (Broeren et al., submitted). That such interactions could have regulatory potential, can be inferred from the observation that preimmunisation in rats with the Vβ18 (17–31) peptide in a suitable adjuvant (DDA) or transfer of the Vβ18 (17–31) specific T cells led to significantly reduced severity of disease upon Mt immunisation. Remarkably enough, also in already established disease, intracutaneous immunisations with the Vβ18 (17–31) peptide led to earlier remission of AA. By administration of TcR peptides in the ear, during the course of AA, positive DTH reactivity was seen at the time of first clinical signs (day 15), for the Vβ18 (17–31) peptide and not for any of the other (control) peptides tested (Broeren et al., submitted). Especially this indicated that T cell responses to V-gene products of disease producing T cells can be part of the natural built-in regulatory mechanisms that contribute to disease remission. In this way, the expression of MHC-class II molecules on activated T cells

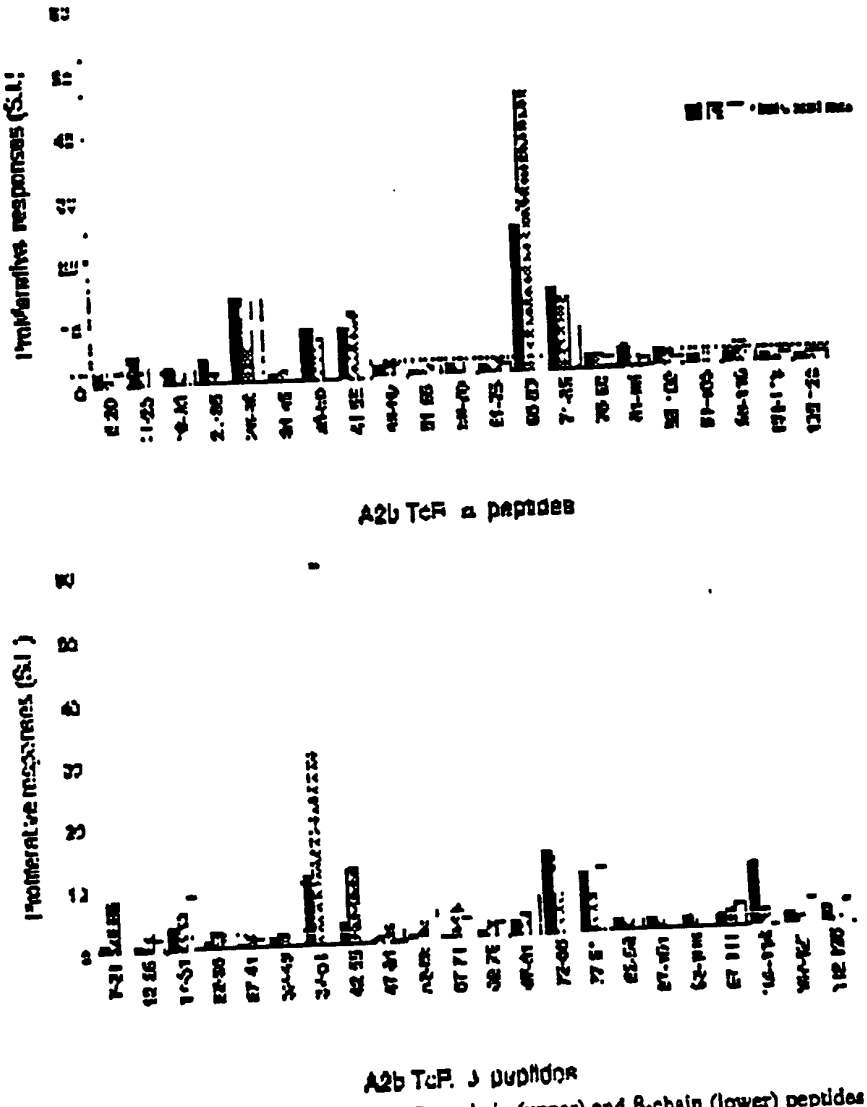

Fig. 3. Proliferative responses to A2b TcR α-chain (upper) and β-chain (lower) peptides in isolated splenocytes after i.p. immunisation and booster with all pooled α- or β-chain peptides. SI=stimulation index

TABLE I

T cell lines used in this study with their specificities and MHC restrictions

T cell line	Specificity	Peptide sequence	Responses to			
			Peptide	rDNA TcR protein	T cell clone	MHC restriction
CDR1 Va11(26–40)	TcR Va11(26–40)	SITTTTVQWFRQNPR	+++	+	-	RT1.B^{l}
CDR2 Va11 (41–55)	TcR Va11(41–55)	GSLINLFYLVPGTKE	++	+	-	RT1.D^{l}
CDR4 Va11 (66–80)	TcR Va11(66–80)	KERYSTLYISNAQVE	+++	+	-	RT1.D^{l}
CDR1 Vb18 (17–31)	TcR Vb18(17–31)	TSLKIQCVVDSQVAL	+++	++	++	RT1.D^{l}
CDRo Vb18 (37–51)	TcR Vb18(37–51)	QFQGQNLMLMATANE	+++	-	-	RT1.D^{l}
CDR4 Vb18 (72–86)	TcR Vb18(72–86)	NLTFSTLTVNNARPE	+++	-	+	RT1.B^{l}
CDRo Vb8.2(43–57)	TcR Vb8.2(43–57)	GLRLIHYSYDVNSTE	+++	++	-	RT1.D^{l}

which is now clear for both human and rat (Broeren et al. 1995a) could well have functional significance, as we have discussed for hsp's, for signalling feed-back regulatory events, in this case targeted to activated T cells. The nature of the regulatory potential of such cells remains to be elucidated. Vβ18 (17–31) responsive cells, which inhibited disease development upon transfer, were seen to produce INFγ and relatively low amounts of IL2. This phenotypic pattern resembles the anergic cells, with supposed suppressive activities, as described by Lombardi et al. (1994). They may consume IL2, which is needed by their target cells and they may compete for MHC-peptide complexes at the APC level. Also, their INFγ production may have suppressive effects, in line with earlier observations that administration of anti-INFγ in the phase of AA remission leads to prolonged disease (Jacob et al. 1989). These experimental observations have proven the principle to be valid: TcR V-gene products are recognizably exposed on T cells and the repertoire harbours T cells with the right specificity, and capacity to respond to them. Even so, TcR fragments may be internalised by professional APCs in order to activate such T cells. These findings have indicated that the prerequisites for preferentially downregulatory control are present in T–T cell recognition events that may occur at the site of inflammation. It is very conceivable that also this contributes to dominant peripheral tolerance.

Fig. 4 represents a compilation of the various T cell specificities which may have contributed to the various arthritis regulatory mechanisms described.

SUMMARY

Tolerance for self has appeared incomplete for many self antigens. We have obtained experimental evidence that both for self heat shock proteins and T cell receptor V-gene products, reactive T cells are part of the normal immune repertoire. Furthermore, it has become apparent that stimulation of T cell responsiveness to these antigens, by using peptide immunisation or by transfer of activated T cells, raises resistance to experimentally induced autoimmune arthritis. In addition, available evidence has suggested that these reactivities may be functional during natural processes of disease remission.

The observations with regard to heat-shock proteins have indicated that mechanisms leading to disease resistance are most efficiently triggered by exposing the immune system to non-self antigens such as bacterial hsp's, which are similar to, but not identical to, self. Experimental evidence has been obtained, that conserved bacterial hsp peptides, may trigger self hsp reactive T cells, with disease suppressive regulatory potential. It is possible that such self hsp reactive T cells, being expanded by recognising bacterial peptides as full agonists, do, in fact, perceive the self epitopes as partial agonists, and therefore have the possibility of displaying downregulatory activity at the site of inflammation. Experiments with peptide analogs of self epitopes, being variants of disease critical T cell epitopes, have indeed

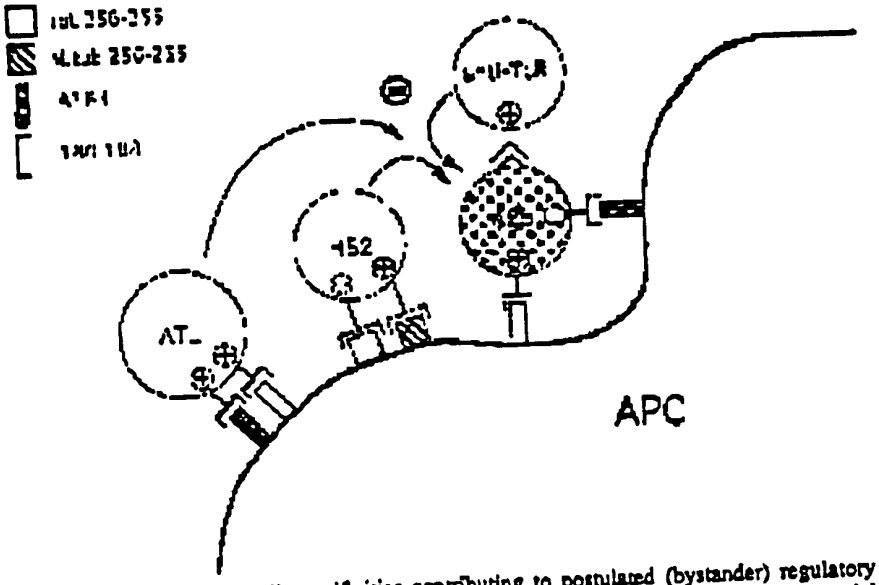

Fig. 4. The various T cell specificities contributing to postulated (bystander) regulatory mechanisms. T cell A2b is the arthritogenic effector cell. A2b is activated by mycobacterial hsp60 180–188. A183 is a non-stimulatory peptide analog of 180–188, which may be a partial agonist for A2b. T cells H.52, ATL and anti-TcR are arthritis suppressive. H.52 recognises mycobacterial 256–265 (full agonist) and the rat homolog peptide 256–265 (partial agonist?). It is proposed that H.52 exerts suppressive effects only after recognition of rat (self) hsp60 256–265. ATL can be activated by A183 ("Competitor-Modulator" peptide analog of 180–188) and by 180–188. Anti-TcR T cell recognises Vbeta18(17–31) as expressed on activated A2b cells. All T cells discussed are MHC class II restricted CD4+ T cells.

suggested that also their activity in modulating disease may comply with the principles of dominant immunological tolerance.

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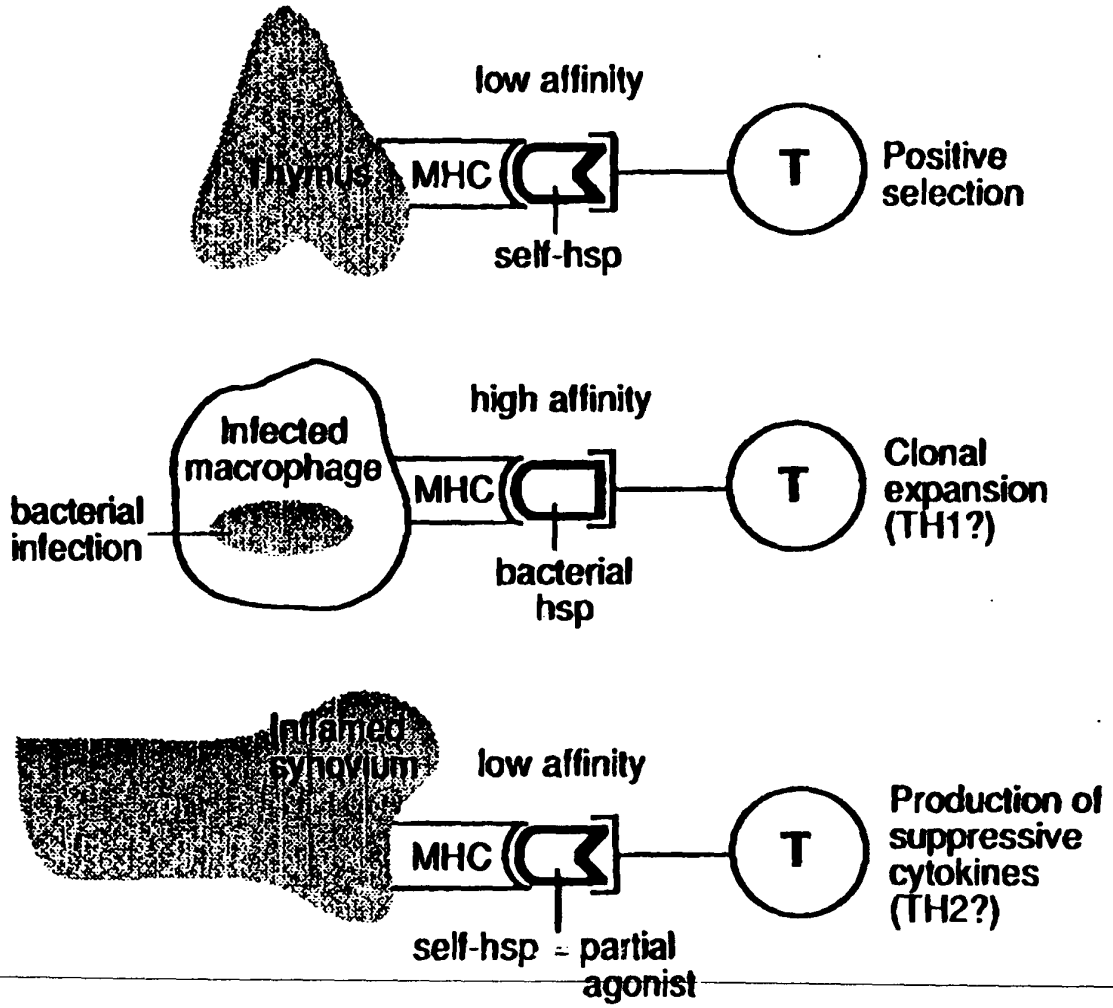
Heat-shock proteins and T cell repertoire



HEAT-SHOCK PROTEINS AND T CELL REPERTOIRE

'Central self-tolerance' (thymus)	Neg. selection for 'full agonist' self-hsp epitope Pos. selection for *'partial agonist'* self-hsp epitope
'Dominant responsiveness' to bacterial hsp's	Expansion of pos. selected cells on *'full agonist'* bacterial hsp epitopes (=conserved [self-crossreactive] hsp epitopes)
'Peripheral tolerance' for conserved self-hsp epitope	(Bystander?) Regulation of inflammation by T cells responding to upregulated *'partial agonist'* self-hsp epitopes (stress-response)

immunogenic antigens.

WO 88/06591 discloses mycobacterial peptides for use in vaccines against mycobacterial infections, including the s quence 231-245 of hsp65 of *M. tuberculosis*. The mapping of T cell epitopes using mycobacterial antigens, including sequence 112-132 of hsp65 is decribed in EMBO J. 6, 1245-1249 (1987). The role of the sequences 211-225, 231-245 and others of mycobacterial hsp65 in human T cell recognition is reported in J. Immunol. 141, 2749-2754 (1988). WO 90/10449 teaches the use of the sequence of the human hsp65 corresponding to the mycobacterial sequence 410-432 in the treatment of insulin dependent diabetes mellitus.

Attempts to induce AA by immunisation with hsp65 alon proved unsuccessful. Instead, this approach conferred r sistance to subsequently attempted induction of AA with whole Mt (5,6). This protective effect is believed to be mediated by T cells specific for hsp65 (7). Preimmunisation with mycobacterial hsp65 has subsequently been reported to confer protection against other forms of experimental arthritis induced with streptococcal cell walls (8), collagen type II (6,9), or synthetic adjuvants such as CP20961 (6) and pristane (10).

Mycobacterial hsp65 belongs to the hsp60 family of heat shock proteins which is highly conserved throughout evolution, and shares 48% aminoacid identity with the mammalian homologue, P1 or hsp60 (11). Expression of mammalian hsp60 is known to be upregulated as a physiological response to various stressful stimuli, and has been shown to be elevated in inflamed synovia of patients with RA (12), or juvenile chronic arthritis (JCA, ref.13).

Description of the invention

The invention is based on the finding that protective epitopes for prevention and treatment of inflammatory diseases are located at relatively short regions (about 5 to 15 aminoacids) of stress proteins, which regions are highly conserved between microorganisms and mammals. In addition to the high degree of identity in the protective epitopes, the proteins are, more generally, highly conserved between microorganisms and mammals.

The term "stress protein" is used here to denote enzymes or proteins that exhibit a raised level of synthesis during inflammation or other stress stimuli in cells residing at the site of such inflammation or stress condition. Normally, stress protein are constitutively expressed, e.g. to exert house-keeping and metabolic functions in cells. In this description, a "microbial stress protein" is to be understood as a microbial homologue of a mammalian stress protein. Inflammation may result from infection, autoimmune disease, tumour growth, transplant rejection or tissue trauma. Other stress stimuli include increased temperature (up to 45°C), drugs, heavy metals, exogenous organic substances, oxidants, bacterial toxins, LPS, stress inducing lipoproteins, mitogens (PHA, ConA) and cytokines, such as IL1, IL2, TNFα, INFα and β, IL6 and IL12.

Raised synthesis can lead to a raised level of excretion or release of such proteins by cells or a raised level of presentation to the immune

Claims

1. Peptide corresponding to a part of the aminoacid sequence of a microbial protein having a conserved mammalian stress protein homologue, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 25%, the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 40%, said part comprising 5-30 aminoacids, at least 5 of which are identical with the corresponding aminoacids in the same relative position in a T cell epitope of said stress protein, said epitope and said part containing at least 4 consecutive aminoacids which are identical with the corresponding mammalian stress protein aminoacids.

2. Peptide according to claim 1, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 40% and the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 50%.

3. Peptide according to claim 1 or 2, wherein said stress protein is selected from heat-shock proteins and stress-induced enzymes.

4. Peptide according to claim 3, wherein said heat-shock protein is heat shock protein hsp65 of *Mycobacterium tuberculosis* (identical to hsp65 of *M. bovis* BCG) as depicted in SEQ ID No. 1.

5. Peptide according to claim 4, wherein the peptide comprises at least 5 aminoacids which are identical with the corresponding aminoacids in the same relative position in one of the sequences 81-100 and 241-270 of SEQ ID No. 1.

6. Peptide according to claim 5, wherein the peptide comprises at least 5 aminoacids which are identical with the corresponding aminoacids in the same relative position in one of the sequences 84-95 and 256-265 of SEQ ID No. 1.

7. [– provisionally deleted –]

8. Peptide according to any one of claims 1–6, wherein said part does not contain one or more sections of 5–50 aminoacids corresponding to T cell epitopes of said stress protein, which epitopes contain less than 3, especially less than 4, consecutive aminoacids which are identical with the corresponding mammalian stress protein amino-acids.

9. Peptide according to any one of claims 1–8, wherein one or more of the aminoacid residues has been exchanged with a residue of an aminoacid having similar size, charge and polarity, or with aminoacid mimetics resulting in one or more backbone modifications.

10. Method of producing a peptide according to any one of claims 1–9, comprising the steps of:

a) selecting a microbial protein having a conserved mammalian stress protein homologue, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 25%, and the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 40%;

b) preparing peptides comprising 5–30 aminoacids at least 5 of which are identical with the corresponding aminoacids in the same relative position in said stress protein, of which a series of at least 4 consecutive aminoacids is identical both to a series of aminoacids of the selected microbial protein and to the corresponding series of mammalian stress protein aminoacids;

c) screening the prepared peptides for the presence of a T cell epitope.

11. Nucleotide sequence encoding a peptide according to any one of claims 1–8.

12. Expression system capable of expressing a peptide according to any one of claims 1–8.

13. Microorganism or eukaryotic cell containing an expression system according to claim 12.

14. T cell or cell expressing a T cell receptor from it, activated by immuno-stimulation using a peptide according to any one of claims 1-9.

15. Antibody raised against a peptide according to any one of claims 1-9.

16. Pharmaceutical composition suitable for treatment of or protection against an inflammatory disease, including autoimmune diseases, such as diabetes, arthritic diseases, atherosclerosis, multiple sclerosis, myasthenia gravis, containing a peptide according to any one of claims 1-9, a nucleotide sequence according to claim 11, an expression system according to claim 12, a cell according to claim 13 or 14, or an antibody according to claim 15.

17. Diagnostic composition suitable for detecting an inflammatory disease, including autoimmune diseases, containing a peptide according to any one of claims 1-9 or an antibody according to claim 15.

Claims

1. Peptide corresponding to a part of the aminoacid sequence of a microbial protein having a conserved mammalian stress protein homologue, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 25%, the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 40%, said part comprising 5-30 aminoacids, at least 5 of which are identical with the corresponding aminoacids in the same relative position in a T cell epitope of said stress protein, said epitope and said part containing at least 4 consecutive aminoacids which are identical with the corresponding mammalian stress protein aminoacids.

2. Peptide according to claim 1, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 40% and the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 50%.

3. Peptide according to claim 1 or 2, wherein said stress protein is selected from heat-shock proteins and stress-induced enzymes.

4. Peptide according to claim 3, wherein said heat-shock protein is heat shock protein hsp65 of *Mycobacterium tuberculosis* (identical to hsp65 of *M. bovis* BCG) as depicted in SEQ ID No. 1.

5. Peptide according to claim 4, wherein the peptide comprises at least 5 aminoacids which are identical with the corresponding aminoacids in the same relative position in one of the sequences 81-100 and 241-270 of SEQ ID No. 1.

6. Peptide according to claim 5, wherein the peptide comprises at least 5 aminoacids which are identical with the corresponding aminoacids in the same relative position in one of the sequences 84-95 and 256-265 of SEQ ID No. 1.

7. [- provisionally deleted -]

8. Peptide according to any one of claims 1-6, wherein said part does not contain one or more sections of 5-50 aminoacids corresponding to T cell epitopes of said stress protein, which epitopes contain less than 3, especially less than 4, consecutive aminoacids which are identical with the corresponding mammalian stress protein amino-acids.

9. Peptide according to any one of claims 1-8, wherein one or more of the aminoacid residues has been exchanged with a residue of an aminoacid having similar size, charge and polarity, or with aminoacid mimetics resulting in one or more backbone modifications.

10. Method of producing a peptide according to any one of claims 1-9, comprising the steps of:

a) selecting a microbial protein having a conserved mammalian stress protein homologue, wherein the overall aminoacid sequence identity between the microbial and the mammalian homologues is at least 25%, and the sequence identity between the microbial and the mammalian homologues of an area of at least 75 consecutive aminoacids is at least 40%;

b) preparing peptides comprising 5-30 aminoacids at least 5 of which are identical with the corresponding aminoacids in the same relative position in said stress protein, of which a series of at least 4 consecutive aminoacids is identical both to a series of aminoacids of the selected microbial protein and to the corresponding series of mammalian stress protein aminoacids;

c) screening the prepared peptides for the presence of a T cell epitope.

11. Nucleotide sequence encoding a peptide according to any one of claims 1-8.

12. Expression system capable of expressing a peptide according to any one of claims 1-8.

13. Microorganism or eukaryotic cell containing an expression system according to claim 12.

14. T cell or cell expressing a T cell receptor from it, activated by immuno-stimulation using a peptide according to any one of claims 1-9.

15. Antibody raised against a peptide according to any one of claims 1-9.

16. Pharmaceutical composition suitable for treatment of or protection against an inflammatory disease, including autoimmune diseases, such as diabetes, arthritic diseases, atherosclerosis, multiple sclerosis, myasthenia gravis, containing a peptide according to any one of claims 1-9, a nucleotide sequence according to claim 11, an expression system according to claim 12, a cell according to claim 13 or 14, or an antibody according to claim 15.

17. Diagnostic composition suitable for detecting an inflammatory disease, including autoimmune diseases, containing a peptide according to any one of claims 1-9 or an antibody according to claim 15.

Fig. 14 (1)

Glyceraldehyde-3-phosphate dehydrogenase sequences of Bacillus stearothermophilus (upper sequence) and Rattus norvegicus (Rat) (lower sequence)

+++ Identical aminoacids: Bacillus / Rat (182 = 54.3%)

\+ Similar, not identical aminoacids: Bacillus / Rat (115)

```
                    5                  10                  15
Ala Val Lys Val Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Asn Val
    +++ +++ +++ +++  +  +++ +++ +++ +++ +++ +++ +++ +++     +++
 -  Val Lys Val Gly Val Asn Gly Phe Gly Arg Ile Gly Arg Leu Val
                     5                  10                  15

            20                  25                  30
Phe Arg Ala Ala Leu Lys Asn Pro Asp Ile Glu Val Val Ala Val Asn
    +++ +++ +++  +   +           +   +   +   +  +++ +++  +  +++
Thr Arg Ala Ala Phe Ser Cys Asp Lys Val Asp Ile Val Ala Ile Asn
                   20                  25                  30

            35                  40                  45
Asp  -  Leu Thr Asp Ala Asn Thr Leu Ala His Leu Leu Lys Tyr Asp
+++     +   +  +++  +  +++     +   +   +   +   +   +  +++ +++
Asp Pro Phe Ile Asp Leu Asn Tyr Met Val Tyr Met Phe Gln Tyr Asp
            35                  40                  45

         50                  55                  60
Ser Val His Gly Arg Leu Asp Ala Glu Val Ser Val Asn Gly Asn Asn
+++  +  +++ +++  +   +   +   +   +  +++  +   +   +   +   +   +
Ser Thr His Gly Lys Phe Asn Gly Thr Val Lys Ala Glu Asn Gly Lys
         50                  55                  60

     65                  70                  75
Leu Val Val Asn Gly Lys Glu Ile Ile Val Lys Ala Glu Arg Asp Pro
+++ +++  +  +++ +++ +++     +++  +   +       +  +++ +++ +++ +++
Leu Val Ile Asn Gly Lys Pro Ile Thr Ile Phe Gln Glu Arg Asp Pro
     65                  70                  75

 80                  85                  90                  95
Glu Asn Leu Ala Trp Gly Glu Ile Gly Val Asp Ile Val Val Glu Ser
    +   +       +++ +++  +      +++  +   +       +++ +++ +++ +++
Val Lys Ile Lys Trp Gly Asp Ala Gly Ala Glu Tyr Val Val Glu Ser
 80                  85                  90                  95

                   100                 105                 110
Thr Gly Arg Phe Thr Lys Arg Glu Asp Ala Ala Lys His Leu Glu Ala
+++ +++     +++ +++  +   +  +++  +  +++  +      +++ +++  +   +
Thr Gly Val Phe Thr Thr Met Glu Lys Ala Gly Ala His Leu Lys Gly
                   100                 105                 110
```

Fig. 14 (2)

```
                115                 120                 125
Gly Ala Lys Lys Val Ile Ile Ser Ala Pro Ala Lys Asn Glu Asp Ile
+++ +++ +++  +  +++ +++ +++ +++ +++ +++  +      +   +       +
Gly Ala Lys Arg Val Ile Ile Ser Ala Pro Ser Ala Asp Ala Pro Met
                115                 120                 125

        130                 135                 140
Thr Ile Val Met Gly Val Asn Gln Asp Lys Tyr Asp Pro Lys Ala His
     +  +++ +++ +++ +++ +++  +   +  +++ +++ +++      +   +
 -  Phe Val Met Gly Val Asn His Glu Lys Tyr Asp  -  Asn Ser Leu
                130                 135                 140

    145                 150                 155
His Val Ile Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Phe
 +   +   +  +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++ +++  +
Lys Ile Val Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu
                145                 150                 155

160                 165                 170                 175
Ala Lys Val Leu His Glu Gln Phe Gly Ile Val Arg Gly Met Met Thr
+++ +++ +++  +  +++  +   +  +++ +++ +++ +++      +++  +  +++ +++
Ala Lys Val Ile His Asp Asn Phe Gly Ile Val Glu Gly Leu Met Thr
        160                 165                 170

                180                 185                 190
Thr Val His Ser Tyr Thr Asn Asp Gln Arg Ile Leu Asp Leu Pro His
+++ +++ +++  +      +++  +   +  +++  +   +   +  +++      +++
Thr Val His Ala Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro Ser
    175                 180                 185

                195                 200                 205
Lys Asp Leu Arg Arg  -  Ala Arg Ala Ala Ala Glu Ser Ile Ile Pro
     +  +++  +  +++      +  +++  +  +++ +++  +   +  +++ +++ +++
Gly Lys Leu Trp Arg Asp Gly Arg Gly Ala Ala Gln Asn Ile Ile Pro
190                 195                 200                 205

                210                 215                 220
Thr Thr Thr Gly Ala Ala Lys Ala Val Ala Leu Val Leu Pro Glu Leu
 +   +  +++ +++ +++ +++ +++ +++ +++  +      +++  +  +++ +++ +++
Ala Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Ile Pro Glu Leu
                210                 215                 220

        225                 230                 235
Lys Gly Lys Leu Asn Gly Met Ala Met Arg Val Pro Thr Pro Asn Val
 +  +++ +++ +++  +  +++ +++ +++  +  +++ +++ +++ +++ +++ +++ +++
Asn Gly Lys Leu Thr Gly Met Ala Phe Arg Val Pro Thr Pro Asn Val
                225                 230                 235

    240                 245                 250
Ser Val Val Asp Leu Val Ala Glu Leu Glu Lys Glu Val Thr Val Glu
+++ +++ +++ +++ +++  +          +++ +++ +++      +   +       +
Ser Val Val Asp Leu Thr Cys Arg Leu Glu Lys Pro Ala Lys Tyr Asp
        240                 245                 250
```

Fig. 14 (3)

```
255                 260                 265                 270
Glu Val Asn Ala Ala Leu Lys Ala Ala Ala Glu Gly Glu Leu Lys Gly
 +   +   +       +   +   +++  +  +++ +++ +++ +++     +++ +++ +++
Asp Ile Lys Lys Val Val Lys Gln Ala Ala Glu Gly Pro Leu Lys Gly
    255                 260                 265

                275                 280                 285
Ile Leu Ala Tyr Ser Glu Glu Pro Leu Val Ser Arg Asp Tyr Asn Gly
+++ +++  +  +++  +  +++  +   +   +  +++ +++     +++  +  +++  +
Ile Leu Gly Tyr Thr Glu Asp Gln Val Val Ser Cys Asp Phe Asn Ser
270                 275                 280                 285

            290                 295                 300
Ser Thr Val Ser Ser Thr Ile Asp Ala Leu Ser Thr Met Val Ile Asp
 +   +      +++ +++ +++  +  +++ +++      +   +   +   +   +   +
Asn Ser His Ser Ser Thr Phe Asp Ala Gly Ala Gly Ile Ala Leu Asn
                290                 295                 300

        305                 310                 315
Gly Lys Met Val Lys Val Val Ser Trp Tyr Asp Asn Glu Thr Gly Tyr
 +   +   +  +++ +++  +   +  +++ +++ +++ +++ +++ +++     +++ +++
Asp Asn Ile Val Lys Leu Ile Ser Trp Tyr Asp Asn Glu Tyr Gly Tyr
            305                 310                 315

    320                 325                 330
Ser His Arg Val Val Asp Leu Ala Ala Tyr Ile Ala Ser Lys Gly
+++  +  +++ +++ +++ +++ +++     +++ +++  +  +++ +++ +++  +
Ser Asn Arg Val Val Asp Leu Met Ala Tyr Met Ala Ser Lys Glu
        320                 325                 330
```

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

12 MAR 1996
PCT

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
BO 39207		
International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NL 95/ 00108	21/03/1995	21/03/1994
International Patent Classification (IPC) or national classification and IPC		
C07K14/35		
Applicant Rijksuniversiteit [RIJKSUNIVERSEIT] UTRECHT et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of – sheets.

3. This report contains indications and corresponding pages relating to the following items:

I ☒ Basis of the report

II ☐ Priority

III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

IV ☐ Lack of unity of invention

V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

VI ☐ Certain documents cited

VII ☒ Certain defects in the international application

VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/10/1995	08.03.96
Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	S.-E. Korsner Telephone No. (+0554)

Form PCT/IPEA/409 (cover sheet) (January 1994) (22/11/1995)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[x] the international application as originally filed.

[ ] the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____,

[ ] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____,

[ ] the drawings, sheets/fig _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:
[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

[ ] the entire international application,

[x] claims Nos. 1-3, 7-17 (when dependent on Claims 1-3)

because:

[ ] the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international preliminary examination (specify):

[ ] the description, claims or drawings (indicate particular elements below) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (specify):

[ ] the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

[x] no international search report has been established for said claims Nos. 1-3, 7-17 (when dependent on Claims 1-3).

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims _____	YES
	Claims 4-17 _____	NO
Inventive Step (IS)	Claims _____	YES
	Claims 4-17 _____	NO
Industrial Applicability (IA)	Claims 4-17 _____	YES
	Claims _____	NO

2. CITATIONS AND EXPLANATIONS

The following documents are referred to:

D1=WO-A-88065916
D2=EMBO Journal, 1987, pp. 1245-1249
D3=Journal of Immunology, 1988, pp. 2749-2754
D4=EP-A-322 990
D5=EP-A-262 710
D6=WO-A-9010449

Novelty (Article 33(2) PCT)

The peptide sequences as claimed, including the more restricted ones of Claim 6, are anticipated by the documents D1-D3.
See especially the following pages:

D1, p.8.....sequence 231-245
D2, p.1247.....sequence 112-132, overlap in DDVAG = sequence 81-85 as claimed - see comment on page 1248,

column 2, about identity.
See also the selection method, page 1248, column 2, bottom.

D3, Table 1....sequences 231-245, 241-255 and 91-105 - the latter overlapping with sequence 84-95 of Claim 6.

Since the related matter of the dependent claims is considered obvious to the skilled man once the sequences are available, no presence of novelty can be acknowledged.

Inventive step (Article 33(3) PCT)

Similar approaches using (other/overlapping) fragments of M. Tuberculosis are already known in the prior art - see also the additional documents D4-D6, and more specifically

D4, p.6.....Use of other fragments for protection against induction of adjuvant arthritis,
D5, p.3.....Use of further fragments, including the neighbouring 171-240, for the preparation of compositions for allevation, treatment and diagnosis of autoimmune diseases.
D6, p.7.....Use of fragments for prevention or treatment of diabetes mellitus.

Having regard to the teachings and the extensive background references of the cited documents, it is evident that most of the characteristics of hsp65 (and related proteins) are well known.
Moreover, the discussion of D3, pages 2752-53, is also instructive about the possibilities offered by these peptides.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Prior art (Rule 5.1(a)(ii) PCT)

The most relevant prior art, as found during the international search, has not been cited in the Description.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1.
On page 5 (bottom) and page 6 (top), the references to Fig. 14 are unclear, because said figure is not present.

2.
Since M. Tuberculosis and M. Bovis are not identical, the bracket of Claim 2 is not clear.
Should the complete sequence be identical, this should be pointed out.

3.
In Claims 5-7, it is not clear whether the wording "..comprise \ at least 5 amino acids which are in the same relative position.." indicates identical amino acids or not.

4.
Claim 9 has no clear interpretation, because the exchange of one or, in particular, more amino acid(s) may result in a completely different peptide, especially in the case of shorter peptides.

5.
Claim 10 borders on a scientific theory under Rule 39.1/67.1 and there is no evidence that this will invariably lead the skilled man to a successful result.
In view of the anticipating prior art, the question of non-unity also arises.

6.
Claim 13 appears to include known microorganisms; in the case of special strains, the conditions of Rule 13bis must be fulfilled (deposits).

TENT COOPERATION TREAT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA 416)
BO 39207		
International application No.	International filing date (day-month-year)	Priority date (day-month-year)
PCT/NL 95/00108	21/03/1995	21/03/1994
International Patent Classification (IPC) or national classification and IPC		
C07K14/35		
Applicant		
RIJKSUNIVERSEIT UTRECHT et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 8 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to nov., inv. step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

CORRECTED VERSION

Date of submission of the demand	Date of completion of this report
04/10/1995	31.05.96
Name and mailing address of the IPEA	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465	S-E Korsner Telephone No. (+8554) S.-E. Korsner

Form PCT/IPEA/409 (cover sheet) (January 1994) (22/11/1995)

Intern. application No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT PCT/NL95/00108

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

[ ] the international application as originally filed.

[x] the description, pages 1, 3-34, as originally filed,
pages _____, filed with the demand,
pages 1a, 2, filed with the letter of 12.02.96,
pages _____, filed with the letter of _____,

[x] the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-17, filed with the letter of 12.02.96,
Nos. _____, filed with the letter of _____,

[x] the drawings, sheets/fig 1-13, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig 14(1)-(3), filed with the letter of 12.02.96,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:
[ ] the description, pages _____.
[ ] the claims, Nos. _____.
[ ] the drawings, sheets/fig _____.

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

[ ] the entire international application,

[x] claims Nos. 1-3, 7-17 (partially)___

because:

[ ] the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international preliminary examination (specify):

[ ] the description, claims or drawings (indicate particular elements below) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (specify):

[ ] the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

[x] no international search report has been established for said claims Nos. 1-3, 7-17 (partially)___.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims 1-17(?) See note below	YES
	Claims	NO
Inventive Step (IS)	Claims 1-17(?) See note below	YES
	Claims	NO
Industrial Applicability (IA)	Claims 1-17	YES
	Claims	NO

2. CITATIONS AND EXPLANATIONS

Novelty and inventive step (Article 33(2) and (3) PCT)

It appears that the present compounds and their use are novel and inventive over the prior art, but the unclarities referred to in Sections VII and VIII must be attended to.

Note:
The search report has been based on a restricted search and refers to Claims 4-6 and to Claims 1-3 and 7-17 in part, including the real examples given in the Description.
This preliminary examination report is established on the same basis.

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

1.
The Description has not been amended to correspond to the amended claims.
See especially the restriction to "5-30 amino acids" (total length of the peptide) and "at least 40% (sequence identity)", both of Claim 1.

Note 1:
Figure 14 was filed 12.02.96 in response to a remark that this figure was not present in the application documents. However, it has later been established that the figure was correctly filed and no objection remains.

Note 2:
Although some amendments have been made in Claim 1, the objection by the ISA to search Claims 1-3 still holds.

Note 3:
There is no Claim 7 [=provisionally deleted].

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

1.
The lengthy definitions, including the use of terms such as "preferably" and "especially" do not meet the requirements for clarity, see e.g. page 3 and claims. In spite of these definitions, the reader would not easily perceive the actual scope of the claims even if he would understand the underlying concept.

Initial objections in view of overlapping sequences disclosed in D1-D3 were rejected by the Applicant on the basis that further criteria were not fullfiled. This may be acknowledged but pinpoints the difficulty encountered by the reader in establishing what is covered by the claims;

D1=WO-A-8806591
D2=EMBO Journal, 1987, pages 1245-1249
D3=Journal of Immunology, 1988, pages 2749-2754

2.
Claim 1, and thus the dependent claims, is now directed to a peptide of 5-30 amino acids.
Even if this may be implicit from line 7, it should preferably be made clear at the beginning of the claim.
The basis for this restriction appears to be page 6, line 4.

3.
Claim 9 is not clear because it appears that further modifications should be made on the peptide.
The final scope is therefore unclear and it would also be doubtful whether further deviations would still result in useful compounds.